PCT / IB 99 / 01465

11. 10. 99

The Patent Office

09/762292

INVESTOR IN PEOPLE

IB 99/1465

AU 1638

Kallis

EJW

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

REC'D 13 OCT 1999

WIPO PCT

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

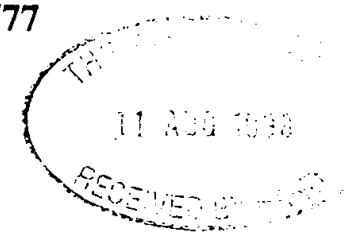
Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed Andrew Gersey

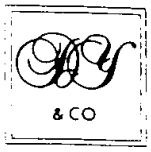
Dated 9 September 1999

Patents Form /77

Patents Act 1977
(Rule 16)



The Patent Office



12AUG98 E382510-17 002246
P01/7700 25.00 - 9817465.9

Request for a grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference — P/5002.GB CTH

2. Patent applicati (The Patent Offi — **9817465.9** — 11 AUG 1998

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Danisco A/S
Langebrogade 1
PO Box 17
DK-1001 Copenhagen K
Denmark

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — Denmark

4. Title of the invention — Selection Method

5. Name of your agent *(if you have one)* — D YOUNG & CO

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

21 NEW FETTER LANE
LONDON
EC4A 1DA

Patents ADP number *(if you have one)* — 59006

6. If you are declaring priority from one or more earlier patent applications, give the country and date of filing of the or each of these earlier applications and (if you know it) the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day/month/year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and filing date of the earlier application

Number of earlier application	Date of filing *(day/month/year)*

8. Is a statement of inventorship and of r[illegible] grant of a patent required in support of this request? (A[illegible] Yes' if:
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d)) Yes

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form

Description 57

Claims(s) 10

Abstract 1

Drawing(s) 1 (2 figures)

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*

Request for preliminary examination and search *(Patents Form 9/77)*

Request for substantive examination *(Patents Form 10/77)*

Any other documents *(please specify)*

11. I/We request the grant of a patent on the basis of this application.

Signature

D YOUNG & CO
Agents for the Applicants

Date

11 08 98

12. Name and daytime telephone number of the person to contact in the United Kingdom

Dr C T Harding

01703 634816

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 01645 500505

b) Write your answers in capital letters using black ink or you may type them

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

SELECTION METHOD

The present invention relates to a selection method.

The present invention also relates to an enzyme and a nucleotide sequence coding for same that are useful in a selection method.

In particular, the present invention relates to a method for the selection (e.g. identification and/or separation) of genetically transformed cells and compounds and genetic material for use in the method.

It is well known that when a nucleotide sequence of interest ("NOI") is to be introduced into a population of cells by transformation, only a certain number of the cells are successfully transformed, i.e., only a certain number of the cells receive the NOI. It is then necessary to identify the genetically transformed cells so that these cells may be separated from the non-transformed cells in the population. For the production of transgenic plants etc., this often requires the use of a selection system that allows the regeneration and growth of the transformed (or transgenic) cells. As these transformed cells frequently constitute a minor fraction of the treated cells, compared to the majority of cells which remain untransformed, so the selection system has to be able to be effective in selecting out the transformed cells.

A common technique for a selection method includes introducing transformed cells and non-transformed cells into a medium that comprises a substance which the transformed cells are able to tolerate. In that medium the transformed cells are able to survive and grow, while the non-transformed cells are prone to growth inhibition and, in some cases, are killed.

Thus, to date, the general strategy has been to introduce a selectable gene along with the NOI(s), and then allowing the transformed cells to survive on selective media while the non-transformed cells are killed (Bowen 1993).

Typically, if a population of plant cells has been subjected to genetic transformation, selection of the transformed cells typically takes place using a selection gene which codes for antibiotic resistance or herbicide resistance. The selection gene is coupled to or co-introduced with the NOI to be incorporated into the plant in question, so that both of the two sequences are incorporated into some or all of the population of cells.

As not all of the cells may have been transformed, the cells are then cultivated on or in a medium containing the respective antibiotic or herbicide to which the genetically transformed cells are resistant by virtue of the selection gene. In this medium, the transformed cells are able to grow and thus be identified out of the total cell population, since the non-transformed cells - which do not contain the antibiotic or herbicide resistance gene in question - have an inhibited growth or even are killed.

So far, the most widely used selectable gene is the neomycin phosphotransferase II (NPTII) gene (Fraley *et al.* 1986) which confers resistance to the aminoglycoside antibiotics kanamycin, neomycin and G-418 (Bevan *et al.* 1983). A number of other selective systems has been developed based on resistance to bleomycin (Hille *et al.* 1986), bromoxynil (Stalker *et al.* 1988), chloramphenicol (Fraley *et al.* 1983), 2,4-dichlorophenoxy-acetic acid (Streber and Willmitzer 1989), glyphosate (Shah *et al.* 1986), hygromycin (Waldron *et al.* 1985) or phosphinothricin (De Block *et al.* 1987).

These selection methods which rely on the use of antibiotics or herbicides suffer from a number of disadvantages. For example, there is concern amongst some people, such as environmental groups and governmental authorities, as to whether it is environmentally safe to incorporate genes coding for antibiotic resistance and/or herbicide resistance into plants and micro-organisms. This concern is of particular significance for food plants and for micro-organisms which are not designed and/or intended to be used in a closed environment (e.g. micro-organisms for use in agriculture), and also for micro-organisms which are designed for use in a closed environment but which may be released from the closed environment.

While such ecological concerns may prove unfounded, as suggested by Flavell *et al.* (1992), they may nevertheless lead to govermental restrictions on the use of antibiotic resistance genes in transgenic plants, and it is therefore desirable to develop new selection methods which are independent of such genes.

In addition, in some or many cases, the corresponding antibiotic or herbicide resistance genes may not be relevant to the desired transgenic trait. Also, they may be undesirable in the final product (Yoder and Goldsbrough 1994).

Thus, the use and subsequent release of selectable genes such as antibiotic resistance genes into the environment has been the target of concern among environmental authorities.

Therefore, new selection systems for the production of transgenic plants without any herbicide or antibiotic resistance genes have been developed, including 3 selection systems based on the concept of 'positive selection' where the transgenic cells have aquired a gene which confers a metabolic advantage to the transgenic cells while starving rather than killing the non-transgenic cells (for a review, see Joersbo 1997).

One of these positive selection methods relates to cytokinins which must be added to obtain optimal shoot regeneration. By adding cytokinin as an inactive glucuronide derivative and using a ß-glucuronidase gene as selectable gene, cells which have acquired this gene by transformation are able to convert the cytokinin glucuronide to active cytokinin while untransformed cells are arrested in development (Joersbo and Okkels 1996; Okkels *et al.* 1997).

Two other selection systems employ, as selectable agents, the carbohydrates mannose and xylose, which are not metabolised by a number of plant species (Bojsen *et al.* 1994). By substituting the normally employed carbohydrate with one of these compounds, cells transformed with a gene encoding an enzyme capable of converting it to a metabolisable isomer are favoured in growth while the non-transgenic cells are starved. Mannose is

initially phosphorylated to mannose-6-phosphate by hexokinase present in plant cells but this compound is not metabolised any further in many plant species. Cells transformed with a phosphomannose isomerase gene are able to convert mannose-6-phosphate to the readily metabolised fructose-6-phosphate, giving these cells a metabolic advantage (Joersbo *et al.* 1998). Xylose can be converted to xylulose by xylose isomerase which functions as the selectable marker in this system (Haldrup 1996).

Another selection system that is not dependent on the use of herbicide and antibiotic resistance genes is described in PCT/GB98/00367. In the general method of PCT/GB98/00367, selectable transformed cells are selected from a population of cells which comprises the selectable genetically transformed cells and possible non-transformed cells. Each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence and optionally a second expressable nucleotide sequence. In the selection method, a component or a metabolic derivative thereof when present in a low concentration in a medium is a nutrient for both the selectable genetically transformed cells and the non-transformed cells. In the selection method, the component or the metabolic derivative thereof when present in a high concentration in a medium is toxic to the non-transformed cells. The first nucleotide sequence codes for a gene product capable of converting the component or the metabolic derivative thereof when present in a high concentration in a medium to a nutrient for the selectable genetically transformed cells. The selection method comprises the step of introducing the population of cells to a medium, wherein the medium optionally comprises a high concentration of the component or the metabolic derivative thereof. In the selection method, the component or the metabolic derivative thereof is a source of both carbohydrate and nitrogen for the selectable genetically transformed cells. Alternatively, in the selection method if a portion of the component serves as a metabolic substrate and is metabolically converted to a derivatised substrate, then that derivatised substrate is capable of providing an allosteric effect on the gene product. In one preferred aspect, the selection method relies on the use of glucosamine.

Despite the advent of selection methods that do not necessarily rely on the use of antibiotic or herbicide resistance genes, it is still desirable to develop new methods for selecting genetically transformed cells or organisms (or parts thereof) comprising such.

According to a first aspect of the present invention there is provided:

a selection method for selecting from a population of cells one or more selectable genetically transformed cells,

wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cells by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to the non-transformed cells;

the method comprising the step of introducing the population of cells to a medium,

wherein the medium comprises the component and/or the metabolic derivative thereof and/or the metabolic precursor therefor and in an amount that is detoxifiable by the transformed cells but in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

According to a second aspect of the present invention there is provided a composition comprising a population of cells comprising selectable genetically transformed cells and possible non-transformed cells; and a medium;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in the medium is detoxifiable by the selectable genetically transformed cells by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof when present in at least a toxic amount in the medium is toxic to the non-transformed cells;

wherein the medium comprises the component and/or the metabolic derivative thereof and/or the metabolic precursor therefor and in an amount that is detoxifiable by the transformed cells but in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

According to a third aspect of the present invention there is provided a population of cells comprising selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cells by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an

enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

According to a fourth aspect of the present invention there is provided a selectable genetically transformed cell comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

According to a fifth aspect of the present invention there is provided a construct for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell;

the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

According to a sixth aspect of the present invention there is provided a vector comprising a construct for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell;

the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

According to a seventh aspect of the present invention there is provided a plasmid comprising a construct for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell;

the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

According to an eighth aspect of the present invention there is provided an organism comprising a selectable genetically transformed cell;

wherein the selectable genetically transformed cell comprises a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

According to a ninth aspect of the present invention there is provided a kit comprising a construct (such as when contained within or on a vector or in a plasmid) for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell; and a medium;

the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof;

wherein the medium comprises the component and/or the metabolic derivative thereof and/or the metabolic precursor therefor and in an amount that is detoxifiable by the transformed cells but in an amount that is toxic to the non-transformed cells.

According to a tenth aspect of the present invention there is provided a plant comprising one or more heterologous enzymes, wherein the heterologous enzymes are any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2).

Here the term "heterologous enzymes" includes enzymes that are derived from a different species or even a different cell. The term also includes homologous enzymes that have been expressed by homologous coding sequences but when under the control of heterologous promoters. Here the term "heterologous promoters" means promoters that are not naturally associated with the coding sequence in question.

According to an eleventh aspect of the present invention there is provided a feed, foodstuff or food prepared from or comprising the aspects of the present invention.

According to a twelfth aspect of the present invention there is provided the use of any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) as a selection means for selecting a genetically transformed cell over a non-transformed cell.

According to a thirteenth aspect of the present invention there is provided the use of any one or more of a nucleotide sequence coding for galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) for providing a selection means for selecting a genetically transformed cell over a non-transformed cell.

According to a fifteenth aspect of the present invention there is provided the use of any one or more of galactose, galactose-1-phosphate, UDP-galactose, or derivatives thereof as a selection means for selecting a genetically transformed cell over a non-transformed cell.

In one preferred aspect, the selectable genetically transformed cell/cells is/are *in vitro* within a culture.

In an alternative preferred aspect, the selectable genetically transformed cell/cells is/are *in vivo* within an organism.

Preferably the selectable genetically transformed cell/cells is/are selectable genetically transformed plant cell/cells.

Preferably an additional nucleotide sequence is present and wherein the additional nucleotide sequence codes for a nucleotide sequence of interest ("NOI").

Preferably the organism is a plant.

Preferably the plant is capable of providing a foodstuff to humans or animals.

Preferably the plant (or part thereof, including cells thereof) is a monocot or a dicot (including legumes).

In a preferred aspect, the plant is any one of rape seed, potato or maize.

In each aspect of the present invention, the metabolic substrate is preferably metabolically converted to a derivatised substrate by the transformed cell.

When the component or the metabolic derivative thereof or metabolic precursor thereof is present in the medium then preferably the component or the metabolic derivative thereof or metabolic precursor thereof is present in an amount that does not detrimentally affect a major proportion of the transformed cells.

Preferably, when the component or the metabolic derivative thereof or metabolic precursor thereof is present in the medium then the component or the metabolic derivative thereof or metabolic precursor thereof is present in an amount that does not detrimentally affect substantially most of the transformed cells.

More preferably, when the component or the metabolic derivative thereof or metabolic precursor thereof is present in the medium then the component or the metabolic derivative thereof or metabolic precursor thereof is present in an amount that does not detrimentally affect substantially all of the transformed cells.

In another embodiment of the present invention the medium comprises a high concentration of the component or the metabolic derivative thereof or metabolic precursor thereof.

However, in some instances the medium of the present invention does not necessarily have to comprise a high concentration of the component or the metabolic derivative thereof or metabolic precursor thereof.

In a further aspect, in some cases the medium need not contain any added quantities of the component or the metabolic derivative or metabolic precursor thereof according to the present invention.

Preferably, however, the medium contains added quantities of the component or the metabolic derivative or metabolic precursor thereof according to the present invention.

Other aspects of the present invention include:

The use of any one or more of galactose, galactose-1-phosphate, UDP-galactose, or any derivative thereof, as a selection means for selecting a genetically transformed cell over a non-transformed cell.

The use of any one or more of a nucleotide sequence coding for any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) for providing a selection means for selecting a genetically transformed cell over a non-transformed cell.

The use of any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) for providing a selection means for selecting a genetically transformed cell over a non-transformed cell.

Preferably, any one or more of the first, second or third expressable nucleotide sequence and/or any one or more of the first, second or third expression product (such as any one or more of the enzymes galactokinase, UTP-dependent pyrophosphorylase, UDP-glucose

dependent uridylyltransferase and UDP-galactose epimerase and/or nucleotide sequences coding for same) are obtainable from any one or more of: *E. coli* (CE Vorgias, H-G Lemaire, KS Wilson 1991. Protein Expr. and Pur. 2, 330-338); *Saccharomyces sp.* (RA Darrow, R Rodstrom 1968. Biochemistry 7, 1645-1654; MA Schell, DB Wilson 1977. J. Biol. Chem. 252, 1162-1166); *Streptomyces coelicolor* A3 (BD Wilson, DS Hogness 1966. in Methods in Enzymol. vol. 8, pp. 229-240, Academic Press, San Diego); *Tetrahymena thermophila* (JE Lavine, E Cantlay, CT Roberts, DE Morse 1982. Biochim. Biophys. Acta 717, 76-85); *Clostridium pasterianum* (F Daldal, J Applebaum 1985. J. Mol. Biol. 186, 533-545); *Kluveromyces lactis* (MI Riley, RC Dickson 1984. J. Bacteriol. 158, 705-712); *Vicia faba*; *Petunia sp.*; and mammals eg. Chinese hampster (B Talbot, J-P Thirion 1982. Int. J. Biochem. 14, 719-725), human (WO 96/09374).

In the above aspects, the phrase "selecting a genetically transformed cell over a non-transformed cell" can be alternatively expressed as "selecting a genetically transformed cell from one or more non-transformed cells".

Thus, according to one aspect of the present invention there is provided a selection system for selecting at least one genetically transformed cell from a population of cells in a medium, wherein the at least one genetically transformed cell is transformed with a nucleotide sequence which encodes an expression product capable of converting a component, that is present in the medium that is at a level that is toxic to non-transformed cells, into a beneficial metabolite for the at least one transformed cell.

The term "cells" is intended to refer to any type of cells from which individual genetically transformed cells may be identified and isolated using the method of the invention. Examples of such cells typically include cells of plants that have a commercial worth - such as crops useful for food or feed production. If desired, other plant cells can be transformed. The term "cells" is also meant to encompass protoplasts, i.e. the protoplasm of a cell enclosed in a membrane but without a cell wall. While it is contemplated that the selection method of the present invention may be used for any type

of cell, the method has been found to be particularly suitable for the selection of genetically transformed plant cells.

The term "population of cells" refers to any group of cells which has been subjected to genetic transformation and from which it is desired to identify those cells which have been genetically transformed and to isolate the genetically transformed cells from non-genetically transformed cells. The population may, for example, be a tissue, an organ or a portion thereof, a population of individual cells in or on a substrate, such as a culture of plant cells, for example a population of cells in a solution or suspension, or a whole organism, such as an entire plant.

The term "selecting" refers to the process of identifying and/or isolating the genetically transformed cells from the non-genetically transformed cells using the method of the present invention.

The term "medium" includes any medium that is capable of sustaining the viability (such as growth) of the transformed cells while inhibiting or killing the non-transformed cells. For example, the medium may comprise typical ingredients of a growth medium but wherein those ingredients are in such an amount that only the transformed cells are selectively grown. In accordance with the present invention, the medium will comprise at least a component, or a metabolic precursor therefor or a derivative thereof, according to the present invention.

The term "toxic" as used herein in relation to the non-transformed means that the component or metabolic derivative thereof or a metabolic precursor thereof has an adverse effect on the non-transformed cells or is metabolised to a derivative that has an adverse effect on the non-transformed cells. An example of an adverse effect is growth inhibition. The term also includes death of the non-transformed cells.

The term "genetically transformed" includes transformation using recombinant DNA techniques.

The term "introducing the population of cells to a medium" means adding the population of cells to the medium or *vice versa*.

The component of the present invention may be derived from a metabolic precursor therefor.

The term "vector" includes expression vectors and transformation vectors.

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

The term "transformation vector" means a construct capable of being transferred from one species to another - such as from an *E.coli* plasmid to an *Agrobacterium* to a plant.

The term "tissue" includes tissue *per se* and organ.

The term "organism" in relation to the present invention includes any organism that could comprise the nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, and/or wherein the nucleotide sequence according to the present invention can be expressed when present in the organism.

Preferably the organism is a plant.

The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, and/or wherein the nucleotide sequence according to the present invention can be expressed within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

Preferably the transgenic organism is a plant.

In a highly preferred embodiment, the transgenic organism (or part thereof) does not comprise the combination of a promoter and at least one of the first nucleotide sequence coding for the first enzyme according to the present invention, the second nucleotide sequence coding for the second enzyme according to the present invention and the third nucleotide sequence coding for the third enzyme according to the present invention, wherein both the promoter and the nucleotide sequence are native to that organism (or part thereof) and are in their natural environment. Thus, in this highly preferred embodiment, the present invention does not cover at least one of a native first nucleotide sequence coding for the first enzyme according to the present invention, a native second nucleotide sequence coding for the second enzyme according to the present invention and a native nucleotide sequence coding for the third enzyme according to the present invention in its natural environment when it is under the control of its native promoter which is also in its natural environment. In addition, in this highly preferred embodiment, the present invention does not cover the native enzyme according to the present invention (namely any one of the first enzyme according to the present invention, the second enzyme according to the present invention, the third enzyme according to the present invention) when it is in its natural environment and when it has been expressed by its native nucleotide coding sequence which is also in its natural environment and when that nucleotide sequence is under the control of its native promoter which is also in its natural environment. In other words, it is preferred that the nucleotide sequence is heterologous to the organism and/or is under the control of a heterologous promoter.

In accordance with a highly preferred aspect of the present invention, the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof are not prepared *in situ* in the medium - i.e. they are actual ingredients that are added to make up the medium.

The term "detoxifiable" means capable of being converted to one or more derivatives that do not have an adverse effect on the transformed cell. An example of an adverse effect is growth inhibition. These derivatives may or may not accumulate in the cells.

In addition, or in the alternative, these derivatives may or may not be fully metabolisied by the transformed cell.

In one aspect of the present invention, the one or more components or metabolic derivatives thereof can act as a nutrient for the transformed cells.

However, in one aspect, the one or more components or metabolic derivatives thereof need not act as the only possible nutrient for the transformed cells. In this regard, the transformed cells may be grown on other suitable growth media which do not contain the one or more components or metabolic derivatives. In this latter case, the transformed cells will not be selectable due to the presence of the sequences of the present invention - but they could be selectable due to the presence of other selectable sequences.

Here, the term "nutrient" includes a substance that is capable of providing directly or indirectly (e.g. *via* a metabolite thereof) energy or atoms that are beneficially useful for maintenance and/or growth and/or reproduction etc. of the cell, tissue, organ or organism.

For example, the term includes a substrate that can be beneficially metabolised and/or beneficially utilised in a metabolic pathway to enable the transformed cells to grow, to proliferate or to be maintained in a viable form.

In accordance with the present invention, a non-transformed cell is a cell that does not comprise at least one of the first nucleotide sequence according to the present invention, the second nucleotide sequence according to the present invention and the third nucleotide sequence according to the present invention.

The non-transformed cell of the present invention may even be a previously transformed cell that does not comprise at least any one of the first nucleotide sequence according to the present invention, the second nucleotide sequence coding according to the present invention and the third nucleotide sequence according to the present invention. The non-

transformed cell of the present invention can, however, contain one or more heterologous nucleotide sequences, or one or more homologous nucleotide sequences under the control of one or more heterologous transcriptional control elements.

The transformed cell of the present invention may contain one or more heterologous nucleotide sequences, or one or more homologous nucleotide sequences under the control of one or more heterologous transcriptional control elements.

In a highly preferred embodiment, the first nucleotide sequence is not in its natural environment. In this regard, the first nucleotide sequence may not be native (i.e. foreign) to the cell or organism. In addition, the first nucleotide sequence may be native to the cell or organism but wherein the first nucleotide sequence is operably linked to a promoter that is heterologous to the first nucleotide sequence.

In accordance with the present invention there may be a plurality of first nucleotide sequences and/or second nucleotide sequences and/or third nucleotide sequences.

The, or each, first nucleotide sequence may be independently selected from DNA or RNA. Preferably, the or each first nucleotide sequence is DNA. More preferably, the or each first nucleotide sequence is recombinant DNA.

The second nucleotide sequence may be independently selected from DNA or RNA. Preferably, the or each second nucleotide sequence is DNA. More preferably, the or each second nucleotide sequence is recombinant DNA.

The third nucleotide sequence may be independently selected from DNA or RNA. Preferably, the or each third nucleotide sequence is DNA. More preferably, the or each third nucleotide sequence is recombinant DNA.

The term "recombinant DNA" means DNA prepared by at least one step that utilises at least one recombinant DNA technique.

Thus, in a first broad aspect the present invention relates *inter alia* to the use of at least one nucleotide sequence encoding an expression product that affects galactose or a metabolic derivative thereof or a metabolic precursor thereof as a selective agent for the selection of transgenic cells, tissues, organs, organisms (such as plants).

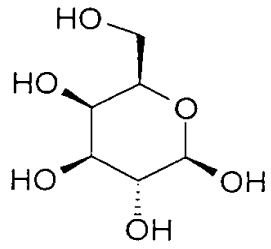
In a second broad aspect the present invention relates *inter alia* to the use of the expression product of at least one nucleotide sequence that affects galactose or a metabolic derivative thereof or a metabolic precursor thereof as a selective agent for the selection of transgenic cells, tissues, organs, organisms (such as plants).

In a third broad aspect the present invention relates *inter alia* to galactose or a metabolic derivative thereof or a metabolic precursor thereof as a selective agent for the selection of transgenic cells, tissues, organs, organisms (such as plants).

Galactose is a hexose which has been demonstrated to be toxic to most plant species (eg. Farkas 1954; Hughes and Street 1974; Roberts et al. 1971).

Galactose has optical isomeric forms and can occur as a linear or a cyclic structure.

An example of a galactose molecule can be pictorially represented as:



Preferably the component is galactose, preferably D-galactose.

If the medium of the present invention comprises a metabolic precursor of galacatose then preferably the metabolic precursor is capable of liberating free galactose or a metabolic derivative thereof.

Preferably the metabolic precursor is capable of liberating free galactose.

More preferably the metabolic precursor is capable of liberating free D-galactose.

If the medium of the present invention comprises a metabolic precursor of galacatose then preferably one or more enzymes are present which can liberate free galactose or a metabolic derivative thereof from the metabolic precursor.

Preferably if the medium of the present invention comprises a metabolic precursor of galacatose then preferably one or more enzymes are present which can liberate free D-galactose.

Prererably the enzyme(s) is at least a galactosidase.

Prererably the enzyme(s) is/are selected from one or more of α-galactosidases or ß-galactosidases.

The enzyme can be present in the medium already or it can be prepared by the medium itself. Alternatively, the enzyme can be prepared by the transformed cell. In this last respect, the transformed cell may also comprise an additional nucleotide sequence coding for an enzyme capable of releasing galactose from a metabolic precursor thereof.

Peferable examples of metabolic precursors of galactose include galactose containing compounds - such as lactoses, melibioses, raffinoses, stachyoses, verbascoses and galactinols.

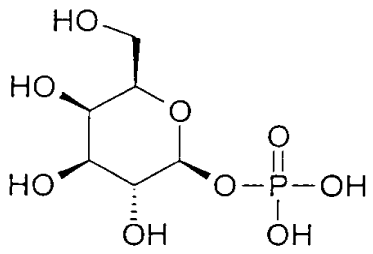
More preferable metabolic precursors of galactose include α-lactose (ß-D-galactopyranosyl [1→4]-α-D-glucose, milk sugar), ß-lactose, melibiose (6-O-α-D-galacto-pyranosyl-D-glucose), raffinose, stachyose, verbascose and galactinol and any other substrate which liberates free D-galactose upon hydrolysis by either α-galactosidases or ß-galactosidases.

Other examples of potentially useful metabolic precursors for use in the galactose selection method if the present invention are chemically derivatised forms of galactose, preferably chemical derivatives of D-galactose, from which free galactose can be liberated by use of appropriate techniques, such as enzyme action. By way of example, suitable chemical derivaties are D-galactose pentaacetate and D-galactose methyl galactoside.

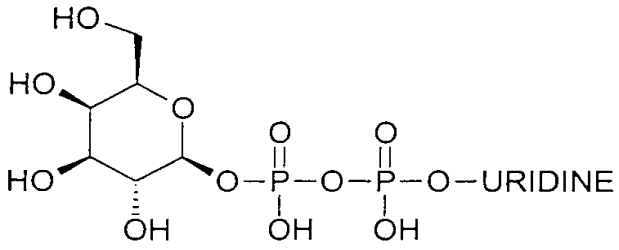
Alternatively or in addition, the medium may comprise a metabolic derivative of galactose.

Preferable examples of such a derivative include galactose-1-phosphate and UDP-galactose.

Galactose-1-phosphate can be pictorially represented as:



UDP-galactose can be pictorially represented as:



More preferable examples of such a derivative include D-galactose-1-phosphate and UDP-D-galactose.

Preferably each of the first expression product; the second expression product and the third expression product is independently selected from galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2).

Preferably each of the first expression product; the second expression product and the third expression product is independently selected from galactokinase (EC 2.7.1.6) obtainable from Petunia, yeast and other microorganisms, *Vicia faba*, *Phaseolus areus*, barley or corn, UTP-dependent pyrophosphorylase (EC 2.7.7.10) obtainable from Petunia, UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12) obtainable from yeast and other microorganisms, UDP-galactose epimerase (EC 5.1.3.2) obtainable from Petunia, yeast and other microorganisms, fenugreek, wheat, sugarcane and various trees.

Preferably, each of the first expressable nucleotide sequence, the second expressable nucleotide sequence and the third expressable nucleotide is independently selected from expressable nucleotide sequences coding for any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2).

Preferably, each of the first expressable nucleotide sequence, the second expressable nucleotide sequence and the third expressable nucleotide is independently selected from expressable nucleotide sequences coding for any one or more of galactokinase (EC 2.7.1.6) obtainable from Petunia, yeast and other microorganisms, *Vicia faba*, *Phaseolus areus*, barley or corn, UTP-dependent pyrophosphorylase (EC 2.7.7.10) obtainable from Petunia, UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12) obtainable from yeast and other microorganisms, UDP-galactose epimerase (EC 5.1.3.2) obtainable from Petunia, yeast and other microorganisms, fenugreek, wheat, sugarcane and various trees.

In one preferred aspect of the present invention, the transformed cells comprise the first expressable nucleotide sequence but not the optional second expressable nucleotide sequence and not the optional third expressable nucleotide sequence.

In this aspect of the present invention, preferably the first expressable nucleotide sequence is a nucleotide sequence coding for UDP-galactose epimerase (EC 5.1.3.2) or UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12). In this aspect, preferably the medium comprises UDP-galactose and/or galactose-1-phosphate, or derivatives thereof.

In one preferred aspect of the present invention, the transformed cells comprise the first expressable nucleotide sequence and the optional second expressable nucleotide sequence, but not the optional third expressable nucleotide sequence.

In this aspect of the present invention, preferably the first expressable nucleotide sequence is a nucleotide sequence that codes for UDP-galactose epimerase, and the

second expressable nucleotide sequence is a nucleotide sequence that codes for UTP-dependent pyrophosphorylase (EC 2.7.7.10) and/or UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12). In this aspect, preferably the medium comprises at least galactose-1-phosphate. For example, the medium may comprise galactose-1-phosphate and UDP-galactose, or derivatives thereof.

In one preferred aspect of the present invention, the transformed cells comprise the first expressable nucleotide sequence and the optional third expressable nucleotide sequence, but not the optional second expressable nucleotide sequence.

In this aspect of the present invention, preferably the first expressable nucleotide sequence is a nucleotide sequence that codes for UDP-galactose epimerase, and the third expressable nucleotide sequence is a nucleotide sequence that codes for galactokinase (EC 2.7.1.6). In this aspect, preferably the medium comprises at least galactose. For example, the medium may comprise galactose and UDP-galactose, or derivatives thereof.

In one preferred aspect of the present invention, the transformed cells comprise the first expressable nucleotide sequence, the optional second expressable nucleotide sequence, and the optional third expressable nucleotide sequence.

In this aspect of the present invention, preferably the first expressable nucleotide sequence is a nucleotide sequence that codes for UDP-galactose epimerase, the second expressable nucleotide sequence is a nucleotide sequence that codes for UTP-dependent pyrophosphorylase (EC 2.7.7.10) and/or UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), and the third expressable nucleotide sequence is a nucleotide sequence that codes for galactokinase (EC 2.7.1.6). In this aspect, preferably the medium comprises at least galactose, or derivatives thereof. For example, the medium may comprise galactose and galactose-1-phosphate, or derivatives thereof. For example, the medium may comprise galactose and UDP-galactose, or derivatives thereof. For

example, the medium may comprise galactose, galactose-1-phosphate and UDP-galactose, or derivatives thereof.

In accordance with the present invention, the medium can comprise one or more of the component, the metabolic derivative thereof and the metabolic precursor.

In order to prepare any one or more of the transformed cells, tissues, organs and organisms according to the present invention, the first expressable nucleotide sequence, the optional second expressable nucleotide sequence and the optional third expressable nucleotide sequence can be introduced into the original non-transformed cells by use of any one or more of a single construct, a single plasmid, or a single vector. Alternatively, the first expressable nucleotide sequence, the optional second expressable nucleotide sequence and the optional third expressable nucleotide sequence can be introduced into the original non-transformed cells by use of any one or more of: two or more constructs, two or more plasmids, or two or more vectors.

In accordance with the present invention any one or more of the first nucleotide sequence, the second nucleotide sequence and the third nucleotide sequence comprises an intron.

In this respect, the presence of the intron within the coding portions of the first nucleotide sequence and/or the second nucleotide sequence and/or the third nucleotide sequence would inactivate the expression products thereof *vis-a-vis* a prokaryote, such as a prokaryotic vector system used to generate the transformed cells, tissue, organs or organism.

Hence, the present invention also covers a process of inactivating a nucleotide sequence or expression product thereof when present in the prokaryote by the insertion of at least one intron into the nucleotide sequence (in particular into the coding portion) thereby inactivating the nucleotide sequence or expression product thereof *vis-à-vis* the prokaryote; and wherein the nucleotide sequence codes for galactokinase (EC 2.7.1.6),

UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or UDP-galactose epimerase (EC 5.1.3.2).

The present invention also covers a prokaryote comprising inactivating a nucleotide sequence or expression product, but wherein the nucleotide sequence comprises at least one intron which inactivates the nucleotide sequence or the expression product thereof in the prokaryote - in particular wherein the intron is present within a coding portion of the gene - wherein the nucleotide sequence codes for galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or UDP-galactose epimerase (EC 5.1.3.2).

With these additional aspects of the present invention, preferably the at least one intron is inserted into a conserved region of the gene, preferably a conserved region within a coding region.

The present invention therefore provides a method for selecting genetically transformed cells - such as cells into which a NOI has been incorporated - by providing the transformed cells with a selective advantage.

The method of the present invention is not dependent on the preparation of genetically transformed plants containing as a selection means a nucleotide sequence coding for antibiotic or herbicide resistance. Nevertheless, the method of the present invention can be used in conjunction with those earlier selection methods should the need arise - if for example it is desirable to prepare cells that have been or are to be transformed with a number of NOIs.

Also, the selection method of the present invention can be used in conjunction with one or more other known selection methods, such as those that are described in WO 93/05163 (the contents of which are incorporated herein by reference) and/or WO 94/20627 (the contents of which are incorporated herein by reference) and/or PCT/GB98/00367 (the contents of which are incorporated herein by reference), should

the need arise - if for example it is desirable to prepare cells that have been transformed with a number of NOIs.

In addition, the selection method of the present invention can be used in conjunction with one or more other selection methods according to the present invention should the need arise - if for example it is desirable to prepare cells that have been transformed with a number of NOIs.

A further beneficial use of a combination of selection methods according to the present invention results in a very efficient multiple screening technique. In this regard, and by way of example, the medium in the first screen utilising the selection method of the present invention would contain added low amounts of the component or the metabolic derivative thereof. With this first screen, selectable transformed cells are selected over at least the majority of the non-transformed cells. Then should - for example - any non-transformed cells be accidentally be carried over in that first screen then a second screen can be carried out. In the second screen the selected population of cells are subjected to a second selection method according to the present invention but wherein the component or the metabolic derivative thereof is present in the medium in a high concentration. In the second screen, predominantly the transformed cells would remain viable.

With this combined aspect of the present invention, the population of cells of the earlier aspects of the present invention can therefore be a pre-selected (e.g. pre-screened) population of cells, wherein the population of cells has been prior selected by one or more selection methods, such as those according to the present invention.

This combined aspect of the present invention can be alternatively expressed as: a selection method for selecting from a population of cells one or more selectable genetically transformed cells, wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells; wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide

sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product; wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cells by action of the first expression product and the optional second expression product and/or the optional third expression product; wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to the non-transformed cells; the method comprising (a) the step of introducing the population of cells to a medium; wherein the medium comprises the component and/or the metabolic derivative thereof and/or the metabolic precursor and in an amount that is detoxifiable by the transformed cells but in an amount that is toxic to the non-transformed cells; wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof; and (b) a subsequent step of introducing at least portion of the transformed cells to a medium that comprises a high concentration of the component or the metabolic derivative thereof or metabolic precursor thereof.

The present invention also encompasses compositions and kits useful for this combined aspect of the present - such as the nucleotide sequence or expression product thereof according to the present invention, a first medium containing no component or metabolic derivative thereof, and a second medium comprising a high concentration of the component or metabolic derivative thereof.

Furthermore, the selection method of the present invention can be used in conjunction (such as sequentially) with further selection methods wherein those further selection methods are a combination of one or more other selection methods according to the present invention and one or more known selection methods - such as those that are

dependent on antibiotic or herbicide resistance and/or those that are disclosed in WO 93/05163 and/or WO 94/20627.

In the selection methods of WO 93/05163 and/or WO 94/20627, the *manA* gene from *Escherichia coli*, which encodes mannose-6-phosphate isomerase (E.C. 5.3.2.8.), was employed as a selectable marker. This selection marker is suitable for *inter alia* the transformation of *Solanum tuberosum*, conferring positive selection in the presence of mannose. In more detail, the coding region of *manA* was ligated into a CaMV 35S expression cassette, and introduced into a binary vector for plant transformation mediated by *Agrobacterium tumefaciens*. To allow comparison of kanamycin selection with selection on mannose, the vector also contained a gene for kanamycin resistance, *nptII*. In order to identify transformants, the construction also contained the *B*-glucuronidase histochemical marker, *uidA*. Stable integration of the *manA* gene was shown by Southern blotting. Extracts from plants transformed with this construct, and selected on mannose, were shown to have specific activities for mannose-6-phosphate isomerase some five hundred fold those of control plants. Expression of *manA* in transformed cells relieved the metabolic paralysis, usually caused by mannose, while also allowing it to serve as a source of carbohydrate for transformants. These effects combined to impose a stringent selection pressure in favour of transformed cells, which allowed the recovery of transformants with a very low frequency of escapes. The percentage of shoots which were shown to be transgenic after selection on mannose was approximately twice that of shoots selected on kanamycin. The transformants selected on mannose have proven to be stable over three generations of plants propagated from tubers.

Hence, the population of cells of the earlier aspects of the present invention can therefore be a pre-selected (e.g. pre-screened) population of cells, wherein the population of cells has been prior selected by one or more selection methods according to the present invention and/or one or more other selection methods.

In addition, or in the alternative, the transformed cells selected by the selection method of the present invention can be subsequently subjected to one or more selection methods according to the present invention and/or one or more other selection methods.

The present invention also provides an expression system that enables transformed cells to be selected by the selection method of the present invention. The expression system can be expressing or can be capable of expressing at least the first nucleotide sequence of the present invention. The expression system may be one or more of a vector, construct, plasmid, cell or organism.

If a cell is also to be transformed with a NOI then the expression system will comprise that NOI - which NOI may be present on or in the same vector, construct, plasmid, cell or organism as the first nucleotide sequence. Alternatively the NOI may be present on or in a different vector, construct, plasmid, cell or organism as the first nucleotide sequence. Preferably, the NOI is present on or in the same vector, construct, plasmid, cell or organism as at least the first nucleotide sequence.

If a cell is to be transformed with one or more NOIs and one or more other genes for one or more other selection methods (such as another selection method according to the present invention and/or a known selection method) those other nucleotide sequences may be present on or in the same vector, construct, plasmid, cell or organism as the first nucleotide sequence. Alternatively one or more of those other nucleotide sequences may be present on or in a different vector, construct, plasmid, cell or organism as at least the first nucleotide sequence. Preferably, those other nucleotide sequences are present on or in the same vector, construct, plasmid, cell or organism as the first nucleotide sequence. This allows for workers to easily prepare and easily select for cells that have been transformed with a number of NOIs etc.

In accordance with the present invention there may be a plurality of NOI(s).

The, or each, NOI may be independently selected from DNA or RNA. Preferably, the or each NOI is DNA. More preferably, the or each NOI is recombinant DNA.

As indicated above, the term "recombinant DNA" means DNA prepared by at least one step that utilises at least one recombinant DNA technique.

The term NOI means any desired nucleotide sequence for incorporation into the cells in question to produce genetically transformed cells. Introduction of nucleotide sequences into, for example, plants is widely practised, and it is believed that there are no limitations upon the nucleotide sequences whose presence may be selected (eg. detected) by use of the selection method of the present invention.

By use of the method of the present invention the presence of the NOI in the genetically transformed cells may be determined without the above-mentioned disadvantages associated with the selection systems relying solely on antibiotic resistance and/or herbicide resistance.

The NOI can be any nucleotide sequence of interest, such as any gene of interest. A NOI can be any nucleotide sequence that is either foreign or natural to the cell or organism (e.g. a particular plant) in question. Typical examples of a NOI include genes encoding proteins and enzymes that modify metabolic and catabolic processes. The NOI may code for an agent for introducing or increasing resistance to pathogens. The NOI may even be an antisense construct for modifying the expression of natural transcripts present in the relevant tissues. The NOI may even code for a compound that is of benefit to animals or humans. Examples of NOIs include nucleotide sequences encoding any one or more of pectinases, pectin depolymerases, polygalacturonases, pectate lyases, pectin lyases, rhamno-galacturonases, hemicellulases, endo-β-glucanases, arabinases, or acetyl esterases, or combinations thereof, as well as antisense sequences thereof. The NOI may encode a protein giving nutritional value to a food or crop. Typical examples include plant proteins that can inhibit the formation of anti-nutritive factors and plant

proteins that have a more desirable amino acid composition (e.g. a higher lysine content than a non-transgenic plant).

The NOI may even code for an enzyme that can be used in food processing such as chymosin, thaumatin and α-galactosidase. The NOI can be a gene encoding for any one of a pest toxin, an antisense transcript such as that for patatin or α-amylase, ADP-glucose pyrophosphorylase (e.g. see EP-A-0455316), a protease antisense, a glucanase or genomic β1,4-endoglucanase.

The NOI may even code for or comprise an intron of a particular gene. Here the intron can be in sense or antisense orientation. In the latter instance, the particular gene could be DNA encoding β-1,4-endoglucanase. Antisense expression of genomic exon or intron sequences as the NOI would mean that the natural β-1,4-endoglucanase expression would be reduced or eliminated but wherein the expression of a β-1,4-endoglucanase gene according to the present invention would not be affected.

The NOI may be the nucleotide sequence coding for the arabinofuranosidase enzyme which is the subject of PCT patent application PCT/EP96/01009 (incorporated herein by reference). The NOI may be any of the nucleotide sequences coding for the ADP-glucose pyrophosphorylase enzymes which are the subject of PCT patent application PCT/EP94/01082 (incorporated herein by reference). The NOI may be any of the nucleotide sequences coding for the α-glucan lyase enzyme which are described in PCT patent application PCT/EP94/03397 (incorporated herein by reference). The NOI may be any of the nucleotide sequences coding for the glucanase enzyme which are described in PCT patent application PCT/EP96/01008 (incorporated herein by reference).

The NOI may also encode a permease or other transport factor which allows the compound or precursor thereof or metabolised derivative thereof to cross the cell membrane and enter the transformed cells. Instead of facilitating uptake of a compound into a cell, the co-introduced nucleotide sequence may alternatively direct the component

or precursor thereof or metabolised derivative thereof to a specific compartment - such as the plasma membrane or into the vacuole or the endoplasmic reticulum.

More than one NOI can be present.

The NOI can be co-introduced with the first nucleotide sequence according to the present invention.

The term "co-introduced" means that the two nucleotide sequences may be coupled to each other, or are otherwise introduced together, in such a manner that the presence of the co-introduced first nucleotide sequence in a cell indicates that the NOI has been introduced into the cell, i.e. if the first nucleotide sequence is shown to have been introduced, the probability that the NOI has also been introduced is significantly increased. The two nucleotide sequences may be part of the same genetic construct and may be introduced by the same vector.

The methods described herein may also be used when the co-introduced first nucleotide sequence and the NOI are introduced independently. This may be performed, for example, by using the same bacteria for incorporation of both genes and incorporating a relatively large number of copies of the NOI into the cells, whereby the probability is relatively high that cells which are shown to express the first nucleotide sequence will also contain and express the NOI.

In order for the introduced first nucleotide sequence and optional NOI to be expressed in the transformed cells, the genetic constructs containing the first nucleotide sequence and/or NOI will typically, but not necessarily, contain regulatory sequences enabling expression of the nucleotide sequences, e.g. known promoters and transcription terminators. Thus, the first nucleotide sequence will typically be associated with a promoter, which may be a constitutive or regulatable promoter, and the NOI will typically also be associated with a constitutive or regulatable promoter.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression.

The promoter could additionally include one or more features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box. The promoters may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the nucleotide sequence of the present invention. For example, suitable other sequences include the *Sh1*-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements.

Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' signal sequence (see Sleat Gene 217 [1987] 217-225; and Dawson Plant Mol. Biol. 23 [1993] 97).

The first nucleotide sequence and/or the second nucleotide sequence and/or the third nucleotide sequence and/or the NOI may comprise one or more introns. In particular, if the first nucleotide sequence and/or the second nucleotide sequence and/or the third nucleotide sequence and/or the NOI encodes an expression product that can detrimentally affect a bacterium and all or a part (e.g. a plasmid thereof or therein) of that bacterium is used either to propagate the NOI or as a means to transform the cells, then it may be desirable for that gene product to be inactive in the bacterium. One way of selectively inactivating the gene product in bacteria is to insert one or more introns into the nucleotide sequence of the first nucleotide sequence and/or the second nucleotide sequence and/or the third nucleotide sequence and/or the NOI, respectively. This intron or those introns would not be removed after transcription in the bacterium but would be so removed in, for example, plants etc.

In a highly preferred embodiment, if the first nucleotide sequence and/or the second nucleotide sequence and/or the third nucleotide sequence and/or the NOI comprises at

least one intron, then that at least one intron is present in a highly conserved region of the first nucleotide sequence or the NOI.

Here, the term "intron" is used in its normal sense as meaning a nucleotide sequence lying within a coding sequence but being removable therefrom.

As mentioned above, the method of the present invention is particularly suitable for the selection of genetically transformed plant cells, thereby allowing identification and isolation of such cells without being essentially dependent on the use of selection genes coding for antibiotic or herbicide resistance.

The selection method of the present invention may be used for selecting cells *in vitro*. However, the selection method of the present invention may also be employed *in vivo* in the sense that it is possible to selectively grow transformed organisms - such as plants - from cells, tissues etc. that comprise the selection system of the present invention.

In vivo use of the selection method of the present invention is of particular importance in connection with genetic transformation performed on whole plants or on plant parts, in which the plants or plant parts comprise both transformed and non-transformed cells, since selection of the transformed cells can, in some instances, be achieved without directly damaging the neighbouring non-transformed cells. For example, in some instances, the transformed cells have a selective advantage compared to the non-transformed cells - such as the ability to still form shoots - but the non-transformed cells suffer in the sense of being damaged or killed, as is the case with using antibiotics or herbicides.

In certain cases, such as when an improved selection frequency is desired, it may be advantageous for the cells to be transformed with a nucleotide sequence that is a selection gene different to the first nucleotide sequence. This additional, selection nucleotide sequence may be an additional gene coding for an enzyme (or other protein or polypeptide) suitable for selection according to the present invention, or it may be a gene

coding for an enzyme (or other protein or polypeptide) for a known selection method, eg coding for resistance to a antibiotic or herbicide or it may be a gene suitable for selection by the selection methods described in WO 93/05163 and/or WO 94/20627. Thus, genetically transformed cells may be selected using a combination of selection techniques. For example, if the transformed cells also possessed genes coding for resistance to at least one antibiotic or herbicide, then the medium could additionally comprise at least one antibiotic or herbicide to which the transformed cells are resistant. In particular, we have found that the medium of the present invention does not impair the effectiveness of the known selection methods that rely on herbicide or antibiotic resistance.

The selective advantage possessed by the transformed cells of the present invention may be any difference or advantage with regard to the non-transformed cells which allows the transformed cells to be readily identified and isolated from the non-transformed cells. This may, for example, be a difference or advantage allowing the transformed cells to be identified by simple visual means, i.e. without the use of a separate assay to determine the presence of a gene that provides the selection means.

As mentioned above, one aspect of the present invention relates to genetically transformed cells which have been selected according to the above method, in particular plant cells, as well as plants, progeny or seeds derived from or derivable from such genetically transformed plant cells. In particular, it is often an advantage that these cells are genetically transformed plant cells whose genome does not contain an introduced (i.e. non-native) nucleotide sequence coding for toxin-resistance, antibiotic-resistance or herbicide-resistance as a selection means. As explained above, there are concerns about whether it is safe to incorporate genes coding for eg antibiotic resistance in eg food plants. Genetically transformed plant cells selected by the method of the present invention which do not contain selection genes for eg antibiotic resistance, as well as plants, progeny and seeds derived from such cells, are therefore clearly advantageous in this respect.

The transformed cells may be prepared by techniques known in the art. For example, if the transformed cells are transformed plant cells reference may be made to EP-B-0470145 and CA-A-2006454.

Even though the selection method according to the present invention is not disclosed in EP-B-0470145 and CA-A-2006454, those two documents do provide some useful background commentary on the types of techniques that may be employed to prepare the transformed plant cells and transgenic plants according to the present invention. Some of these background teachings are now included in the following commentary.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries a first nucleotide sequence or construct according to the present invention and which is capable of introducing the nucleotide sequence or construct into the genome of an organism, such as a plant.

The vector system may comprise one vector, but it can comprise at least two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* (An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. *et al.* (1980), *Tissue Culture Methods for Plant Pathologists,* eds.: D.S. Ingrams and J.P. Helgeson, 203-208).

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above.

The first nucleotide sequence or construct of the present invention should preferably be inserted into the Ti-plasmid between the border sequences of the T-DNA or adjacent a T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct. Preferably, the vector system is an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

In the construction of a transgenic plant the promoter or nucleotide sequence or construct of the present invention may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful microorganism is *E. coli.*, but other microorganisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli.* it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the first nucleotide

sequence or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the promoter or nucleotide sequence or construct of the invention, which DNA is subsequently transferred into the plant cell to be modified.

Naturally, the present invention is not limited to just the use of *Agrobacterium* systems to transform plants. In this regard, other suitable techniques may be used - such as electroporation and/or particle bombardment (biolistics).

As reported in CA-A-2006454, a large number of cloning vectors are available which contain a replication system in *E. coli* and a selection means which allows a selection of the transformed cells. The vectors contain for example pBR322, the pUC series, the M13 mp series, pACYC 184 etc. In this way, the promoter or nucleotide or construct of the present invention can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the transformation in *E.coli*. The *E.coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered and then analysed - such as by any one or more of the following techniques: sequence analysis, restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted or selectively amplified by PCR techniques and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid.

After each introduction method of the first nucleotide sequence or construct according to the present invention in the plants the presence and/or insertion of further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema, in: The Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblasserdam, 1985, Chapter

V; Fraley, *et al.*, Crit. Rev. Plant Sci., 4:1-46; and An et al., EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists,* eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). With this technique, infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a root, a stem or another part of the plant.

Typically, with direct infection of plant tissues by *Agrobacterium* carrying the first nucleotide sequence or the construct, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium.* The inoculated plant or plant part is then grown on a suitable culture medium.

When plant cells are constructed, these cells are grown and, optionally, maintained in a medium according to the present invention following well-known tissue culturing methods - such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc, but wherein the culture medium comprises a component according to the present invention. Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting the transformed shoots and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Further teachings on plant transformation may be found in EP-A-0449375.

Reference may even be made to Sprngstad *et al* (1995 Plant Cell Tissue Organ Culture 40 pp 1-15) as these authors present a general overview on transgenic plant construction.

In a highly preferred embodiment, the present invention is based on our finding that it is possible to use constructs comprising an expressable nucleotide sequence gene coding for any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) to prepare transformed cells wherein the transformed cells can be selected from non-transformed cells.

In addition, the present invention also covers transgenic plants comprising the transformed cells or constructs of the present invention.

Thus, in a highly preferred embodiment the present invention covers transgenic plants comprising transformed cells or constructs that comprise an expressable nucleotide sequence gene coding for any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2).

In order to explain in more detail these highly preferred aspects of the present invention, reference shall be made to at least Figures 1 - 2, in which

Figure 1 is a schematic diagram of a metabolic pathway; and

Figure 2 is a schematic diagram of a metabolic pathway.

Galactose metabolism in plants have been studied in Petunia which is one of the very few plant species being able to use galactose as carbon source to sustain growth (Dressler *et al.* 1982). The first step of galactose metabolism in Petunia as well as in yeast and other microorganisms is the phosphorylation of galactose to galactose-1-phosphate by galactokinase (EC 2.7.1.6) (see Figs. 1 and 2 below). The next step is the conversion to UDP-galactose which in Petunia can be performed by either a UTP-dependent pyrophosphorylase (EC 2.7.7.10) or a UDP-glucose-dependent uridylyltransferase (EC

2.7.7.12) while in microoganisms only or predominantly the latter reaction occurs. The UDP-galactose is finally converted to UDP-glucose by UDP-galactose epimerase (EC 5.1.3.2). Thus, the conversion of galactose to a glucose-containing metabolite is irreversible.

The reason for the toxicity of galactose on plant cells is believed to be due to the absence of one or more of the enzymatic activities required for the conversion of galactose to UDP-glucose. Thus, in order to use galactose as selective agent, the transformed plant cells must be supplemented with the lacking gene(s) so that enzymatic activities for the entire pathway from galactose to UDP-glucose are present.

However, it is likely that many plant species do not require the insertion of all 3 genes in the Leloir pathway as the absence of just one gene in this pathway will render the plant tissue sensitive to galactose.

The first enzyme in galactose metabolism is galactokinase which has been reported to be present in some plants such as *Vicia faba* (Dey 1983), *Phaseolus areus* (Chan and Hassid 1975; Neufeld *et al.* 1960), barley and corn (Roberts *et al.* 1971). Roberts *et al.* (1971) studied the accumulation of galactose containing metabolites in corn roots fed with toxic levels of galactose and they found that free galactose and galactose-1-phosphate each accounted for close to 50% of the indentifiable metabolites. These data were in accordance with the hypothesis that the causal agent of galactose toxicity may be galactose-1-phosphate. As galactose is toxic to most plant species, this in turn implies that many plant species have galactokinase activity.

The next enzyme in the galactose metabolism is UTP-hexose-1-phosphate uridyltransferase, which has only been observed in rare cases such as Petunia, or UDP-glucose uridylyltransferase.

In contrast to the two preceeding enzymes, the last enzyme, UDP-galactose epimerase, has been reported in a range of plant species such as fenugreek (Clermont and Percheron

1979), wheat (Fan and Feingold 1969), sugarcane (Maretzki and Thom 1978) and various trees (Dalessandro and Northcote 1977) in addition to the above mentioned species. The reason for the widespread occurrence of this enzyme is presumably related to the fact that its main function is the production of UDP-galactose from UDP-glucose for the synthesis of cell wall components.

Thus, in order to enable transformed plant tissue to metabolise galactose, it is likely that it is sufficient for some plant species to introduce just one gene eg. a UTP-hexose-1-phosphate transferase gene or a UDP-glucose uridylyltransferase gene which would then function as the selectable gene. However, for other plant species it may be required to introduce two or even three genes involved in the metabolism of galactose. From a technical point of view, it presents no problems to use more than one gene as selectable gene.

Although a galactose selection system also employs a carbohydrate as selective agent, it does not resemble 'positive selection' due to the high toxic effect of galactose.

The selection system described here is based on enabling transformed plant cells to metabolise D-galactose. This D-galactose can be supplied to the plant cells or tissues during selection as free D-galactose or as a D-galactose containing compound. Examples of such D-galactose containing compounds are α-lactose (ß-D-galactopyranosyl [1→4]-α-D-glucose, milk sugar), ß-lactose, melibiose (6-O-α-D-galacto-pyranosyl-D-glucose), raffinose, stachyose, verbascose and galactinol which all liberate free D-galactose upon hydrolysis by either α-galactosidases or ß-galactosidases which are present in a wide range a plant species and tissues.

Other examples of potentially useful compounds for galactose selection are chemically derivatized D-galactose such as D-galactose pentaacetate and methyl galactoside, from which free D-galactose can be produced by the action of appropiate enzymes present in plant cells.

It can also envisaged that the galactose selection system can be modified so that galactose-1-phosphate can be used as selective agent, in which case the selectable gene is most likely to be an UTP-hexose-1-phosphate uridyltransferase gene or an UDP-glucose uridylyltransferase gene.

Yet another modification of the galactose selection system relates to the addition of other carbohydrates or other substances to the selection medium which affect the toxicity of galactose. Furthermore, it will also be obvious for those skilled in the art that changes in the employed galactose concentration in the course of the selection can affect selection efficiency.

Thus, the present invention provides a new selection method based on the novel use of galactose and/or a metabolic derivative thereof and/or a metabolic precursor thereof.

As galactose and the genes encoding the enzymes responsible for the metabolism of galactose are well-characterized, we believe that galactose would be suitable for use as a selective agent for the selection of transgenic cells, tissues, organs, organisms (such as plants), provided that the transgenic cells, tissues, organs, organisms (such as plants) aquire the appropiate genes for metabolising, detoxifying and/or tolerating galactose.

The present invention will now be described only by way of examples.

TRANSFORMATION STUDIES

The following examples demonstrate that the genes coding for galactokinase (EC 2.7.1.6) (*galK*), UTP-dependent pyrophosphorylase (EC 2.7.7.10) (*galP*), UDP-glucose-dependent uridyl transferase (EC 2.7.7.12) (*galT*), UDP-galactose epimerase (EC 5.1.3.2) (*galE*) gene can be used as a means to provide selection of transformed cells, such as transgenic potato or maize shoots, on or in media containing galactose or a derivative thereof as a selective agent. For convenience, the term *galX* has been used to denote any one or more of *galK*, *galP*, *galT*, *galE*.

TRANSGENIC POTATO PLANTS

General teachings on potato transformation may be found in our copending patent applications PCT/EP96/03053, PCT/EP96/03052 and PCT/EP94/01082 (the contents of each of which are incorporated herein by reference).

For the present studies, the following protocol was adopted.

Plasmid construction

The disarmed *Agrobacterium tumefaciens* strain LBA 4404, containing the helper *vir* plasmid pRAL4404 (Hoekema *et al*, 1983 Nature 303 pp 179-180), was cultured on YMB agar ($K_2HPO_4.3H_2O$ 660 mg l^{-1}, $MgSO_4$ 200 mg l^{-1}, NaCl 100 mg l^{-1}, mannitol 10 g l^{-1}, yeast extract 400 mg l^{-1}, 0.8% w/v agar, pH 7.0) containing 100 mg l^{-1} rifampicin and 500 mg l^{-1} streptomycin sulphate. Transformation with a plasmid containing *galX* under the control of a plant expressable promoter (such as the E35S promoter) was accomplished using the freeze-thaw method of Holters *et al* (1978 Mol Gen Genet 163 181-187) and transformants were selected on YMB agar containing 100 mg l^{-1} rifampicin and 500 mg l^{-1} streptomycin, and 50 mg l^{-1} gentamycin sulphate. The resultant plasmid is called pVICTOR IV 35S *galX*. Transformation with a control construct lacking the *galX* gene was performed in the same manner. In addition, the T-DNA will include a screenable marker gene encoding a screenable marker enzyme, such as the *β-glucuronidase* gene from *E. coli*, driven by a plant expressable promoter. This facilitates the optimization of the methods as transgenic cells/shoots/plants are more easily found using this marker (Jefferson *et al* 1987, EMBO J, 6:3901-3907).

Transformation of plants

Shoot cultures of *Solanum tuberosum* cv Saturna were maintained on LS agar containing Murashige Skoog basal salts (Sigma M6899) (Murashige and Skoog (1965) Physiol. Plant. 15: 473-497) with 2 μM silver thiosulphate, and nutrients and vitamins as

described by Linsmaier and Skoog (1965 Physiol. Plant. 18 100-127). Cultures were maintained at 25°C with a 16 h daily photoperiod. After approximately 40 days, subculturing was performed and the shoots cut into segments of approximately 8 mm length.

Shoot cultures of approximately 40 days maturity (5-6 cm height) were cut into 8 mm internodal segments and/or leaves were cut off and wounded by making 2-4 small cuts over the midrib of the leaf. These were then placed into liquid LS-medium containing *Agrobacterium tumefaciens* transformed with pVICTOR IV 35S *galX* (A_{660} = 0.5, pathlength 1 cm). Following incubation at room temperature for 30 minutes, the segments were dried by blotting on to sterile filter paper and transferred to LS agar (0.8% w/v containing 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 500 μg l^{-1} trans-zeatin. The explants were covered with filter paper, moistened with LS medium, and covered with a cloth for three days at 25°C. Following this treatment, the segments can be washed with liquid LS medium containing 800 mg l^{-1} carbenicillin, and then transferred on to LS agar (0.8% w/v) containing 1 mg l^{-1} trans-zeatin, 100 mg l^{-1} gibberellic acid (GA3), with sucrose (eg 10-20 g l^{-1}). This agar contains galactose (eg such as in an amount of from about 0.5 - 5.0 g l^{-1}).

The segments were sub-cultured to fresh substrate each 3-4 weeks. In 3 to 4 weeks, shoots develop from the segments and the formation of new shoots continued for 3-4 months.

The regenerated shoots are maintained on substrate composed of LS-substrate, 0.002 mM silver thiosulphate and agar (8.0 g/l). Carbenicillin (800 mg/l) can be added if desired.

The transgenic plants may be verified by performing a *β-glucuronidase* assay on the leaf tips of the surviving shoots according to Hodal *et al.* (Plant. Sci. (1992), 87: 115-122).

Alternatively, the transgenic genotype of the regenerated shoot may be verified by performing NPTII assays (Radke, S. E. *et al*, Theor. Appl. Genet. (1988), 75: 685-694) or by performing PCR analysis according to Wang *et al* (1993, NAR 21: 4153-4154).

The shoots (height approximately 2-3 cms) were transplanted from rooting substrate to soil and placed in a growth chamber (21°C, 16 hour light 200-400$\mu E/m^2/sec$).

When the plants were well established they were transferred to the greenhouse, where they were grown until tubers had developed and the upper part of the plants were senescing.

Harvesting

The potatoes were harvested after about 3-6 months and then analysed.

The transformed shoots can be distinguished from the non-transformed shoots by adding galactose to their substrate of leaf tips cultured in in-vitro. After harvest of the shoots, the transformed shoots can be selected by adding amounts of galactose to the shoot medium. The transformed shoots will be resistent to galactose and will survive as opposed the non-transformed shoots which will be inhibited in growth.

Analysis of Transformants

In order to confirm the integration of *galX*, genomic DNA may be isolated by the method of Dellaporta *et al* (1983 Plant Mol Biol Rep 1 19-21) and samples of this DNA, digested with *EcoR*I, subjected to electrophoresis in an 0.8% w/v agarose gel and transferred to Hybond N+ membranes (Amersham) by Southern blotting (Southern, 1975 J Mol Biol 98 503-517). Probes for the coding region of *galX* may be used as templates for random primed synthesis of ^{32}P-labelled probe after the method of Feinberg and Vogelstein (1983 Anal Bioch 137 266-267) and hybridised to the Southern blots at high stringency (65°C, 0.1 x SSC).

Selection of transgenic shoots was accomplished using a selection medium according to the present invention.

Transgenic shoots are obtained on selection media according to the present invention indicating that the selection medium can be varied significantly and remain useful for the selection of transgenic shoots.

TRANSGENIC MAIZE PLANTS

Introduction

Since the first publication of production of transgenic plants in 1983 (Leemans, 1993 Biotechnology 11 s22), there have been numerous publications of production of transgenic plants including especially dicotyledon crop plants.

Until very recently there were very few reports on successful production of transgenic monocotyledononary crop plants. This relatively slow development within monocots were due to two causes. Firstly, until the early 1980s, efficient regeneration of plants from cultured cells and tissues of monocots had proven very difficult. This problem was ultimately solved by the culture of explants from immature and embryogenic tissue, which retain their morphogenic potential on nutrient media containing plant growth regulators. Secondly, the monocots are not a natural host for *Agrobacterium tumefaciens*, meaning that the successful developed techniques within the dicots using their natural vector *Agrobacterium tumefaciens* was unsuccessful for many years in the monocots.

Nevertheless, it is now possible to successfully transformation and produce fertile transgenic plants of maize using methods such as: (1) Silicon Carbide Whiskers; (2) Particle Bombardment; (3) DNA Uptake by PEG treated protoplast; or (4) DNA Uptake in Electroporation of Tissue. Each of these methods - which are reviewed by Thompson

(1995 Euphtytica 85 pp 75-80) - may be used to prepare *inter alia* transgenic maize according to the present invention.

In particular, the particle Gun method has been successfully used for the transformation of monocots. However, EP-A-0604662 reports on a different method of transforming monocotyledons. The method comprises transforming cultured tissues of a monocotyledon under or after dedifferentiation with *Agrobacterium* containing a super binary vector as a selection means a hygromycin-resistant gene was used. Production of transgenic calli and plant was demonstrated using the hygromycin selection. This method may be used to prepare *inter alia* transgenic maize according to the present invention.

Subsequent to the method of EP-A-0604662, EP-A-0672752 reports on non-dedifferentiated immature embryos. In this regard, both hygromycin-resistance and PPT-resistance genes were used as the selection means, with PPT giving rise to 10% or more independent transformed plants. This method may be used to prepare *inter alia* transgenic maize according to the present invention.

To date, it would appear that transgenic maize plants can be successfully produced from easily-culturable varieties - such as the inbred line A188. In this regard, see the teachings of Ishida *et al* (1996 Nature Biotechnology 14 pp 745-750). The method disclosed by these workers may be used to prepare *inter alia* transgenic maize according to the present invention.

Vasil (1996 Nature Biotechnology 14 pp 702-703) presents a further review article on transformation of maize.

Even though it is possible to prepare transformed maize by use of, for example, particle Gun mediated transformation, for the present studies the following protocol is adopted.

Plasmid construction

The same protocol as outlined above is adopted. In this respect, the protocol also uses pVICTOR IV 35S *galX*. Likewise, transformation with a control construct lacking the *galX* gene was performed in the same manner.

Isolation and cocultivation of explants

Immature embryos of, for example, maize line A188 of the size between 1.5 to 2.5 mm were isolated and cocultivated with *Agrobacterium tumefaciens* strain LBA 4404 in N6-AS for 2-3 days at 25°C under illumination. Thereafter, the embryos were washed with sterilized water containing 250 mg/l of cefotaxime and transferred to an LS medium and 250 mg/l cefotaxime and galactose in concentrations of up to 100 mg/l (the medium is hereafter called LSS2).

Conditions for the selection of transgenic plants

The explants were cultured for three weeks on LSS2 medium and then transferred to an LS medium containing galactose and optionally cefotaxime. After three weeks on this medium, green shoots were isolated and tested for *β-glucuronidase* (Jefferson *et al* 1987, EMBO J 6:3901-3907) activity.

Rooting of *β-glucuronidase* positive shoots

β-glucuronidase positive shoots were transferred to an MS medium containing 2 mg/l for rooting. After four weeks on this medium, plantlets are transferred to pots with sterile soil for acclimatisation.

Selection of transgenic shoots was accomplished using a selection medium accoding to the present invention.

After four weeks the shoots were harvested and all explants were transferred to fresh selection medium (same composition) and after another four weeks of selection the last shoots were harvested.

After harvest, the shoots were analysed for GUS activity using the histochemical assay.

Transgenic shoots are obtained on selection media according to the present invention indicating that the selection medium can be varied significantly and remain useful for the selection of transgenic shoots.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

REFERENCES

Bevan M, Flavell RB and Chilton MD. 1983. Nature 394, 184-187.

Bojsen K, Donaldson I, Haldrup A, Joersbo M, Kreiberg JD, Nielsen J, Okkels FT, Petersen SG. 1994. Mannose and xylose based positive selection. WO94/20267.

Bowen BA. 1993. In 'Transgenic Plants' Vol. 1. Eds.: Kung SD and Wu R. Academic Press ISBN 0-12-428781-6, pp. 89-146.

Chan PH and Hassid. 1975. Anal. Biochem. 64, 372-379.

Clermont S and Percheron F. 1979. Phytochem. 18, 1963-1965.

Dalessandro G and Northcote DH. 1977. Biochem J. 162, 281-288.

De Block M, Botterman J, Vandewiele M, Docky J, Toen C, Gossele V, Movva NR, Thompson C, Van Montagu M and Leemans J. 1987. EMBO J. 6, 2513-2518.

Dressler K, Biedlingmaier S, Grossberger H, Kemmer J, Nolle U, Rodmanis-Blimer A and Hess, D. 1982. Z. Pflanzenphysiol. 107, 409-418.

Dey PM. 1983. Eur. J. Biochem. 136, 155-159.

Fan D-F and Feingold DS.1969. Plant Physiol. 44, 599-604.

Farkas GL. 1954. Biol. Zentralblatt 73, 506-521.

Flavell RB, Dart E, Fuchs RL and Fraley RT. 1992. Bio/Technology 10, 141-144.

Fraley RT, Rogers SG and Horsch RB. 1986. CRC Critical Reviews in Plant Science 4, 1-45.

Fraley RT, Rogers SG, Horsch RB, Sanders P, Flick J, Adams S, Bittner M, Brand L, Fink C, Fray J, Galluppi G, Goldberg S. and Woo S. 1983. Proc. Natl. Acad. Sci. USA 80, 4803-4806.

Haldrup A. 1996. Ph. D. Thesis, University of Copenhagen, Denmark.

Hille J, Verheggen F, Roelvink P, Franssen H, Kammen AV and Zabel P. 1986. Plant Mol. Biol. 7, 171-176.

Hughes R and Street HE. 1974. Ann. Bot. 38: 555-564.

Joersbo M. 1997. Antobiotic resistance genes and transgenic plants. Nordic Seminar, Oslo, ISBN 82-91683-04-2 pp. 69-78.

Joersbo M and Okkels FT. 1996. Plant Cell Rep16, 219-221.

Joersbo M, Donaldson I, Pedersen SG, Brunstedt J and Okkels FT. 1998. Mol. Breeding, 4: 111-117.

Maretzki A and Thom M. 1978. Plant Physiol. 61, 544-548.

Neufeld EF, Feingold DS and Hassid WZ. 1960. J. Biol. Chem. 235, 906-909.

Okkels FT, Ward JL and Joersbo M. 1997. Phytochem. 46, 801-804.

Roberts RM, Heishamn A and Wicklin C. 1971. Plant Physiol. 48, 36-42.

Shah DM, Horsch RB, Klee HJ, Kishore GM, Winter JA, Tumer NE, Hironaka CM, Sanders PR, Gasser CS, Aykent S, Siegel NR, Rogers SG and Fraley RT. 1986. Science 233, 478-481.

Stalker DM, McBride KF and Malyj LD. 1988. Science 242, 419- 423.

Streber WR and Willmitzer L. 1989. Bio/Technology 7, 811-816.

Waldron C, Murphy EB, Roberts JL, Gustafson, GD, Armour SL and Malcolm SK. 1985. Plant Mol. Biol. 5, 103-108.

Yoder JI and Goldsbrough P. 1994. Bio/Technol. 12, 263-267.

CLAIMS

1. A selection method for selecting from a population of cells one or more selectable genetically transformed cells,

wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cells by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to the non-transformed cells;

the method comprising the step of introducing the population of cells to a medium,

wherein the medium comprises the component and/or the metabolic derivative thereof and/or the metabolic precursor and in an amount that is detoxifiable by the transformed cells but in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an

enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

2. A composition comprising a population of cells comprising selectable genetically transformed cells and possible non-transformed cells; and a medium;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in the medium is detoxifiable by the selectable genetically transformed cells by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof when present in at least a toxic amount in the medium is toxic to the non-transformed cells;

wherein the medium comprises the component and/or the metabolic derivative thereof and/or the metabolic precursor and in an amount that is detoxifiable by the transformed cells but in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an

enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

3. A population of cells comprising selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cells by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

4. A selectable genetically transformed cell comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

5. A construct for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell;

the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

6. A vector comprising a construct for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell;

the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

7. A plasmid comprising a construct for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell;

the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

8. An organism comprising a selectable genetically transformed cell;

wherein the selectable genetically transformed cell comprises a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an

enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

9. A kit comprising a construct (such as when contained within or on a vector or in a plasmid) for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell; and a medium;

the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cell by action of the first expression product and the optional second expression product and/or the optional third expression product;

wherein the component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to a non-transformed cell;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof; and

wherein the component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof;

wherein the medium comprises the component and/or the metabolic derivative thereof and/or the metabolic precursor and in an amount that is detoxifiable by the transformed cells but in an amount that is toxic to the non-transformed cells.

10. The invention according to any one of the preceding claims wherein the selectable genetically transformed cell/cells is/are *in vitro* within a culture.

11. The invention according to any one of the preceding claims wherein the selectable genetically transformed cell/cells is/are *in vivo* within an organism.

12. The invention according to any one of the preceding claims wherein the selectable genetically transformed cell/cells is/are selectable genetically transformed plant cell/cells.

13. The invention according to any one of the preceding claims wherein an additional nucleotide sequence is present and wherein the additional nucleotide sequence codes for a nucleotide sequence of interest.

14. A plant prepared from or comprising the invention according to any one of the preceding claims, preferably wherein the plant is capable of providing a feed, foodstuff to humans or animals.

15. A plant according to claim 14 wherein the plant is any one of potato or maize.

16. A plant comprising one or more heterologous enzymes, wherein the heterologous enzymes are any one or more of galactokinase (EC 2.7.1.6), UTP-dependent

pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2).

17. A foodstuff or food prepared from or comprising the invention according to any one of the preceding claims.

18. Use of any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) as a selection means for selecting a genetically transformed cell over a non-transformed cell.

19. Use of any one or more of a nucleotide sequence coding for galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) for providing a selection means for selecting a genetically transformed cell over a non-transformed cell.

20. A prokaryote comprising a nucleotide sequence coding for galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2); wherein the nucleotide sequence comprises at least one intron which inactivates the nucleotide sequence or the expression product thereof in the prokaryote.

21. Use of any one or more of galactose, galactose-1-phosphate, UDP-galactose, or a derivative thereof, as a selection means for selecting a genetically transformed cell over a non-transformed cell.

22. A selection method or transformed cell, tissue, organ, or organism (or part thereof) substantially as described herein.

ABSTRACT

SELECTION METHOD

A selection method for selecting from a population of cells one or more selectable genetically transformed cells is described. The population of cells comprises selectable genetically transformed cells and possible non-transformed cells. Each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product. A component or a metabolic derivative thereof or a metabolic precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cells by action of the first expression product and the optional second expression product and/or the optional third expression product. The component or the metabolic derivative thereof or the metabolic precursor thereof can be present in an amount in a medium that is toxic to the non-transformed cells. The method comprising the step of introducing the population of cells to a medium. The medium comprises the component and/or the metabolic derivative thereof and/or the metabolic precursor and in an amount that is detoxifiable by the transformed cells but in an amount that is toxic to the non-transformed cells. Each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a metabolic derivative thereof or a metabolic precursor thereof. The component and/or the metabolic derivative thereof and/or the metabolic precursor thereof is galactose or a metabolic derivative thereof or a metabolic precursor thereof.

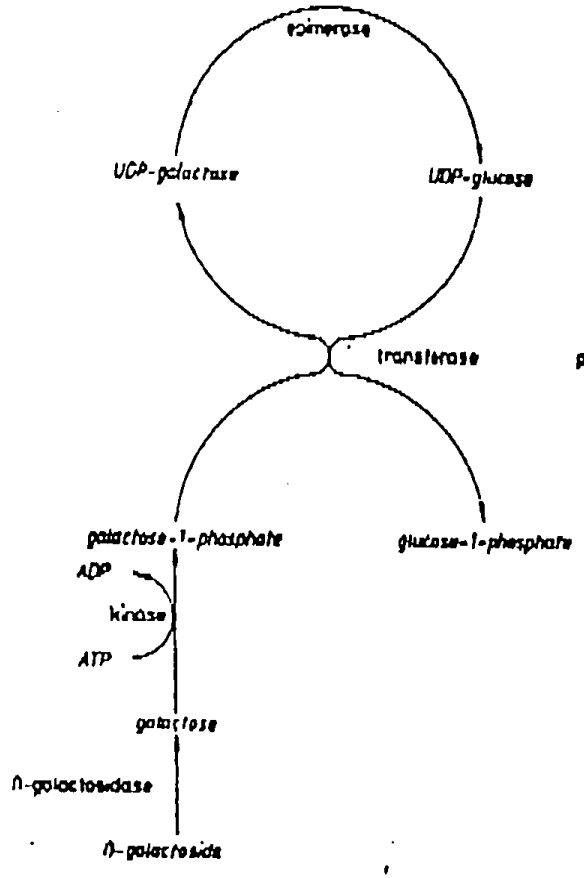


Fig. 1: Leloir pathway of galactose metabolism.

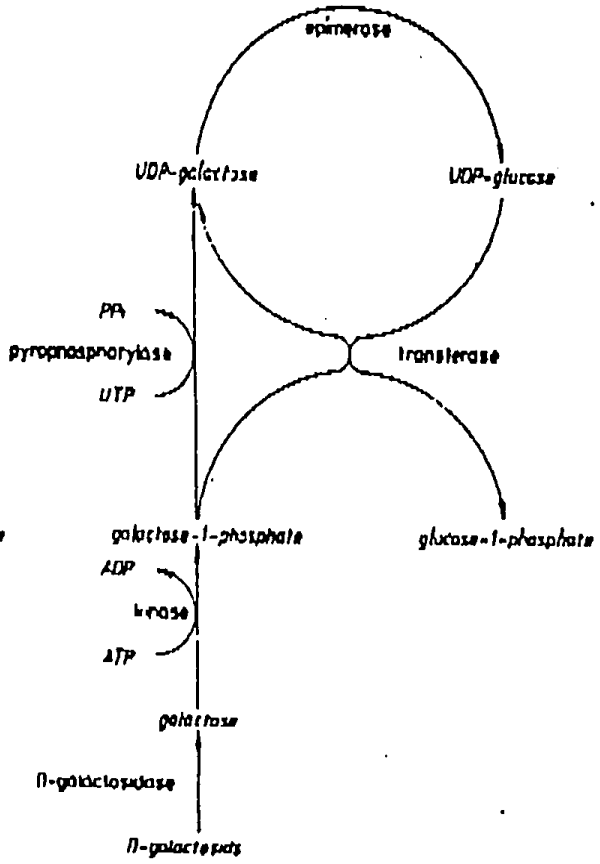


Fig. 2: Petunia galactose metabolism.

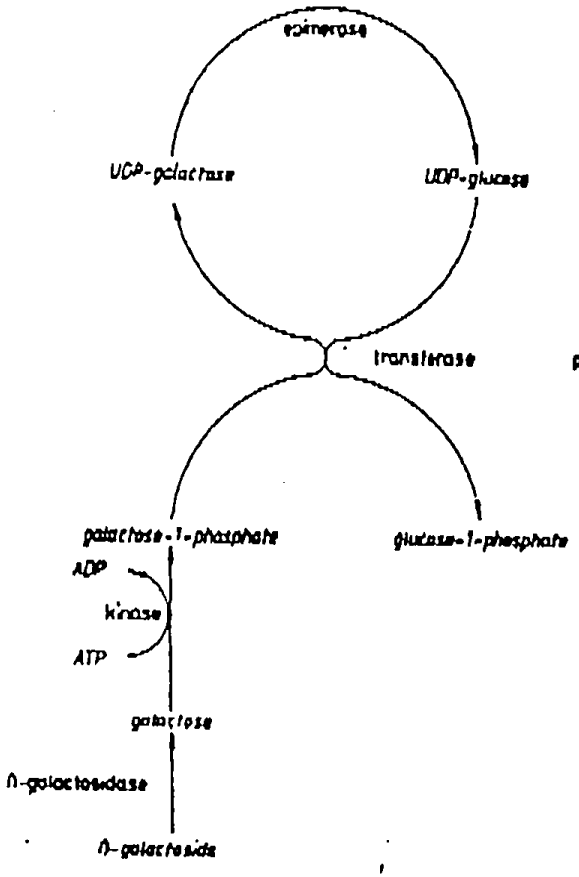


Fig. 1: Leloir pathway of galactose metabolism.

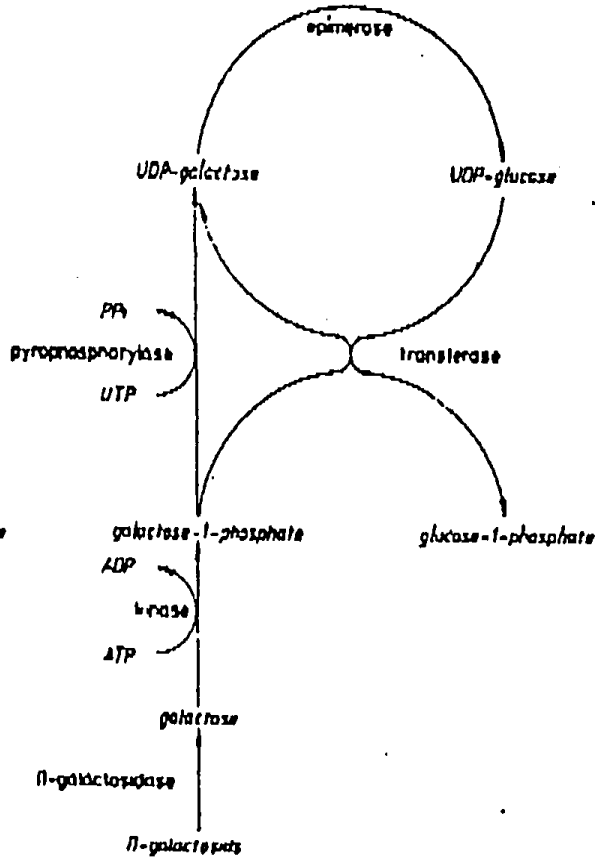


Fig. 2: Petunia galactose metabolism.